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TITLE: SIRT3 Is a Mitochondrial Tumor Suppressor and Genetic Loss Results in a Murine Model for ER/PR-Positive Mammary Tumors Connecting Metabolism and Carcinogenesis

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14. ABSTRACT The goals of this synergistic project are to establish the mitochondria localized sirtuin protein SIRT3 as a tumor suppressor in breast cancer and to define its role as a molecular link between aging and breast cancer. <i>Sirt3</i> knockout mice develop ER/PR positive breast tumors later in life. These ER/PR-positive tumors are histologically similar to breast tumors common in old women. In humans, loss of SIRT3 is seen in a significant fraction of breast cancers and may serve as a molecular biomarker. Molecular targets of SIRT3 deacetylation have been identified, including MnSOD and OSCP. Antibodies that recognize specific acetylated lysine residues targeted by SIRT3 in these molecules have been identified and validated and are being developed as potential novel biomarkers in breast cancer. These studies have enhanced our understanding of the molecular links between aging and breast cancer and provide novel potential biomarkers of breast cancer in humans.				
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INTRODUCTION

SIRT3 is a mitochondrial deacetylase that appears to play a central role in regulating the acetylation of target proteins in response to cell stress and nutrient distress (Park et al., 2011; Tao et al., 2010). SIRT3 has been proposed to function in maintaining mitochondrial integrity and to serve as a bona fide tumor suppressor (Haigis et al., 2012). Deletion of Sirt3 in mice results in the development of breast cancers (Kim et al., 2010). One third of female Sirt3 knockout mice developed mammary gland tumors by 24 months. These tumors coexpress the estrogen and progesterone receptors (ER/PR), paralleling the well-differentiated, receptor-positive tumor type commonly seen in breast malignancies in older women. Analysis of human tumor mRNA expression databases showed a significant reduction in SIRT3 mRNA in breast cancers compared to benign tissue as well as an association with grade (Kim et al., 2010). Initial immunohistochemistry studies confirmed loss of SIRT3 protein expression in human breast tumors as well. These results suggest that SIRT3 is a human tumor suppressor and support the need for further studies to examine SIRT3 and its targets as potential biomarkers in human breast cancer.

BODY

Statement of Work - Task 1 - Identify Sirt3 mitochondrial deacetylation targets and determine if these targets are regulated by extracellular stimuli known to activate sirtuin function (e.g., resveratrol). These targets will subsequently be knocked down (with siRNA) to determine if there is a mechanistic connection between the increase in superoxide and the stress-induced genomic instability observed in SIRT3^{-/-} cells (months 1-18).

Results: The results for Task 1 are detailed in the report for W81XWH-10-1-0661. These studies have identified Manganese Superoxide Dismutase (MnSOD) and OSCP as Sirt3 targets. These studies point to the regulation of ATP production in the mitochondria as a process that is regulated by reversible acetylation of lysine residues of key SIRT3 targets. The specific lysine residues in MnSOD and OSCP proteins targeted by Sirt3 have been identified and antibodies developed. Figure 1 shows western blot analysis validating the OSCP K139-acetyl antibody. SIRT3 deacetylates K139 in an NAD-dependent manner, thereby reducing antibody detection of the acetyl K139 antigen. Figure 2 shows increases in acetylated OSCP-K139, MnSOD-K68 and MnSOD-K122 in Sirt3 knockout mouse livers. These antibodies will be next validated for use in immunohistochemistry, especially using formalin-fixed paraffin-embedded tissue so we can use then in the analysis of mouse and human tumors. We will correlate expression of the antigens detected by these antibodies with loss of Sirt3 and determine the utility of these new antibodies to serve as additional biomarkers in breast cancer.

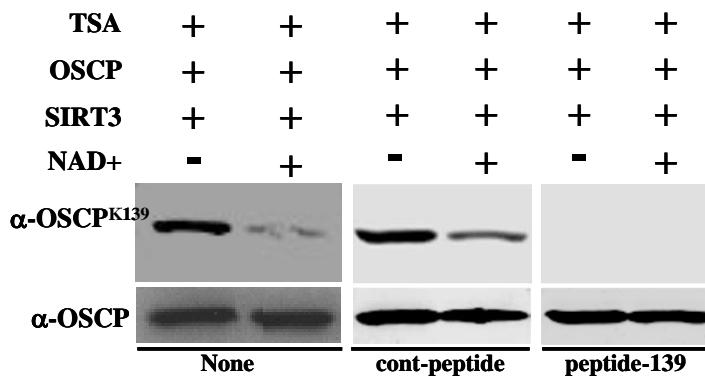


Figure 1. Validation of the OSCP lysine 139 anti-acetyl antibody. Flag-tagged OSCP is expressed in HEK 293T cells treated with TSA (1 μ M). Flag-OSCP was then immunoprecipitated, incubated with purified SIRT3 protein with or without NAD. Mixtures were blotted with anti-OSCP acetyl 139 lysine antibody (Epitomics, Inc, - The Rabbit Monoclonal Antibody Company, Burlingame, CA). Identical experiments were performed with the K139 acetylated peptide (Ac-peptide) or a control non-acetylated K139 peptide (Cont-peptide).

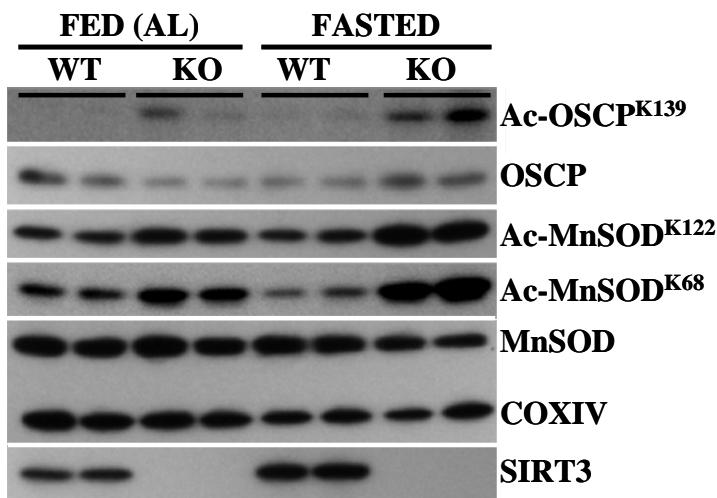


Figure 2: Livers from 3 month old Sirt3 WT or KO mice fasted for 48 hours were harvested. Mitochondrial extracts were blotted with the antibodies indicated. Note increased K139-OSCP, K122-MnSOD and K68-MnSOD acetylation.

Statement of Work - Task 2 - Determine if exposure to resveratrol or overexpression of a MnSOD gene will prevent increases in ROS in MEFs and/or decrease the development of mammary tumors in Sirt3 knockout mice and transformation in SIRT3^{-/-} MEFs (months 7-24).

Results: The mice for this study are being generated by W81XWH-10-1-0661 while W81XWH-10-1-0662 will be involved in the analysis of the mammary tumors in Sirt3 knockout mice. These studies are slated to start in month 7 and results are therefore expected in the second year of the project. The W81XWH-10-1-0661 report has detailed the breeding schema for generating Sirt3 knockout mice as well as the power analysis that guides the number of mice needed to detect the expected decrease in tumor incidence.

Statement of Work - Task 3 - Determine if loss of SIRT3 ductal protein in ER/PR-positive and -negative breast samples from the Vanderbilt Breast Spore correlates with clinically significant outcomes including response to therapy, local tumor control, disease free survival, and overall survival (months 1-24).

In this task we proposed to conduct H & E stained of human samples or slides that will be reviewed to assess the diagnosis and grade, in a blinded manner with no prior knowledge of IHC results. In addition, we will determine clinicopathologic variables including age, tumor grade, stage, mitotic count, and ER and PR status and these will be assessed by response to therapy, local tumor control, disease free survival, and overall survival. These endpoints will be determined via Kruskal-Wallace non-parametric analysis of variance will be done to determine how marker expression varies with grade. The log-rank test will be used to evaluate the statistical significance of disease-free survival by the Kaplan-Meier method for univariate analysis. Finally, log-plots will be used to estimate survival function to test the proportional hazards assumption. The Wald test will be used for Cox proportional hazards regression analysis, and data will first be tested to ensure they meet the assumptions for using the Cox test.

This work is ongoing and has been slightly delayed to accommodate the validation of the new antibodies discussed in the “statement of work – task 1 section” that may be molecular biomarkers. In addition, new results from D. Gius and others indicate that *Sirt3* deficient cells have increased HIF-1 α expression leading to cellular metabolic reprogramming (Warburg effect). Thus HIF-1 α expression will be included in our analysis.

KEY RESEARCH ACCOMPLISHMENTS:

1. Sirt3 is a mitochondrial tumor suppressor is hypothesized to play a role in the development of ER/PR+ breast malignancies.
2. Development of antibodies for SIRT3 lysine substrates in MnSOD and OSCP.

REPORTABLE OUTCOMES:

1. Antibodies for SIRT3 lysine substrates in MnSOD and OSCP validated by western blot analyses

CONCLUSION:

Breast cancer incidence increases with age, but the basis for this association is not understood. SIRT3 may provide a molecular link between breast cancer and aging. This synergistic DOD idea award has facilitated the development of a Sirt3 knockout mouse model of aging-related ER+ breast cancers as well as new potential biomarkers for breast cancer. The completion of these studies should provide important new insights into breast tumorigenesis as well as provide possible new therapeutic and prognostic targets.

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